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Identification of yeasts by hydrolysis of amides

Bestimmung von Hefen durch Amidhydrolyse

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Key words. Yeasts, identification, amides, amidases.

Schlüsselwörter. Hefen, Identifizierung, Amide, Amidasen.

Summary. We analysed the hydrolysis of 23 amides by 500 yeast and yeast-like strains isolated from clinical specimens, identified to species level by conventional methods, in order to verify the validity of this method of species identification. The results show that 10 of these amides (acetamide, acrylamide, alaninamide, formamide, glycinamide, propionamide, urea, thioacetamide, thiourea and valeramide) are sufficient to differentiate seven genera and 19 species, with an occasional requirement for three additional tests: cycloheximide susceptibility, surface film formation on liquid medium and ascospore formation. The study of the amidase activity in yeasts and yeast-like fungi seems to be a promising method of identifying strains isolated from clinical samples.

Zusammenfassung. Es wurde eine Reihe von 23 Amiden an 500 aus klinischen Proben gewonnenen Hefenstämmen geprüft, deren Specieszugehörigkeit nach konventionellen Methoden bestimmt worden war, um die Brauchbarkeit der Amidhydrolyse zur Differenzierung verschiedener Hefearten zu bewerten. Die Ergebnisse zeigen, daß 10 dieser Amide (Acetamid, Acrylamid, Alaninamid, Formamid, Glycinamid, Propionamid, Thioacetamid, Thioharnstoff, Harnstoff und Valeramid) bereits ausreichen, um die Gesamtheit von 500 Stämmen 7 Gattungen und 10 Arten zuzuordnen. In einigen Fällen wurde als zusätzliches Merkmal die Hautbildung in flüssigem Medium, die Toleranz gegenüber Cycloheximid und die Produktion von Ascosporen benutzt. Die Amidhydrolyse erweist sich in unserer Studie als vielversprechendes System zur Identifizierung von Hefearten klinischer Herkunft.

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Introduction

In view of the progressive increase in number and diversity of yeasts involved in human pathology, a highly complex taxonomy is necessary to identify new opportunistic species. At present, standard identification processes are based upon colony and cell morphology, tolerance of cycloheximide, growth at different temperatures, hydrolysis of urea, reduction of nitrate and, above all, the fermentation and assimilation of carbon compounds [1–8]. Enzymatic activity on various substrates [9–13] and resistance to dyes and fungicides [14, 15] have recently been used to identify yeasts. The adoption of micromethods of identification has proved a major step forward in terms of availability and efficiency, since such methods encompass a variety of tests, including assimilation tests and some fermentation, enzymatic and biochemical tests. With these methods an acceptable degree of characterization may be achieved, although not all perform equally well. The most widely known is the API 20 C Yeast Identification System (BioMérieux, France) [16, 17], which many authors take as the standard against which other systems are compared. Some amides other than urea have been used successfully to differentiate *Cryptococcus* species, making it possible to establish a new taxonomic criterion for this genus [18]. There are, however, no references in the literature to the application of this method to other genera of yeasts or yeast-like fungi. Our purpose in this study was, thus, to examine the usefulness of amide

hydrolysis for the identification of yeast species of clinical interest.

Material and methods

The yeasts used in this study were taken from patients treated in 1992 at the Hospital Universitario "Puerta del Mar", in Cádiz (Spain). A total of 500 different strains isolated from 454 samples taken from 449 patients were tested. The strains were kept frozen at −20 °C in brain–heart infusion broth (Difco Laboratories, Detroit, NJ, USA) containing 15% glycerine. Taxonomic characterization of the strains was performed by examining their morphological (colony morphotype, formation of a surface film on the liquid medium, blastesis, capsule production, hyphae/pseudohyphae, chlamydospores, arthrospores and ascospores), physiological (growth at different temperatures and in 50% glucose, tolerance to cycloheximide), biochemical (hydrolysis of urea, nitrate reduction) and nutritional (fermentation and assimilation of carbon compounds) characteristics. All these determinations include six control strains: *Candida albicans* ATCC 752, *Candida glabrata* ATCC 2001, *Candida krusei* ATCC 24408, *Candida parapsilosis* ATCC 7330, *Candida tropicalis* ATCC 750 and *Cryptococcus neoformans* ATCC 2344. We examined amidase activity against 23 amides, at a concentration of 0.25% in a solid medium (urea agar base according to Christensen, E. Merck, Darmstadt, Germany), with the exception of urea, which was used in the same medium at the normal concentration of 2%. The amides tested in this work were as follows: acetazolamide, acrylamide, L-alaninamide HBr, glycinamide HCl, isonicotinamide, niacinamide, L-phenylalaninamide, pyrazinamide, salicylamide, DL-tryptophanamide, L-tyrosinamide (Sigma, St Louis, MO, USA); benzamide, malonamide, succinamide, valeramide (Fluka Chemie, Buchs, Switzerland); allylurea, 2-chloroacetamide, propionamide (Merck, Schuchardt, Germany); acetamide, formamide, thioacetamide, thiourea (Merck, Darmstadt, Germany); and urea (Difco Laboratories).

The amide solutions were prepared in distilled water, sterilized by Millipore filter (pore diameter 0.45 µm), added to the sterile medium in aseptic conditions and cooled to 50–55 °C. The tubes were inclined such that the medium formed a slope some 3 cm in length. The target strains were cultivated for 48 h in Sabouraud glucose agar (Difco) before preparation of a suspension in sterile saline solution of level 2 on the McFarland scale of turbidity. The tubes were inoculated with 100 µl of suspension and then incubated at 30 °C for 10 days. Readings were taken at 5, 7 and 10 days. Amide-free medium was used as control for each strain and assay. All strains were assayed twice to check repeatability. A positive reaction was recorded by the appearance of a more or less pronounced pink colour resulting from the alkalization of the medium by ammonia released from hydrolysis of the amide.

Results

None of the strains displayed activity against 13 amides (Table 1). These were acetazolamide, allylurea, benzamide, chloracetamide, isonicotinamide, malonamide, niacinamide, phenylalaninamide, pyrazinamide, salycilamide, succinamide, tyrosinamide and tryptophanamide. The most frequently hydrolysed amide was propionamide, which was hydrolysed by 97% of the strains tested, while acrylamide and thiourea were hydrolysed by only 1% of strains. Table 2 shows the 500 strains according to genus and species. Strains were identified as belonging to seven genera and 19 species, three species being teleomorphs of *Candida kefyr* (*Kluyveromyces marxianus*), *Candida pelliculosa* (*Pichia anomala*) and *Candida valida* (*Pichia membranaefaciens*). Table 2 also shows which strains had amidase activity against the 10 compounds that were hydrolysed by any of the strains: acetamide (ACE), acrylamide (ACR), alaninamide (ALA), formam-

Table 1. The 23 amides tested and their frequency of utilization by 500 yeast strains

Amide	%
Acetamide	27.8
Acetazolamide	0
Acrylamide	1
L-Alaninamide HBr	15.4
Allylurea	0
Benzamide	0
2-Chloroacetamide	0
Formamide	27.2
Glycinamide HCl	9.6
Isonicotinamide	0
Malonamide	0
Niacinamide	0
L-Phenylalaninamide	0
Propionamide	97
Pyrazinamide	0
Salicylamide	0
Succinamide	0
Thioacetamide	1.2
Thiourea	1
DL-Tryptophanamide	0
Tyrosinamide	0
Urea	3.6
Valeramide	25.4

Table 2. Amidasic patterns of 500 yeast strains, obtained from a 10-amide test

Pattern	Species	Positive strains	%	Amide									
				ACE	ACR	ALA	FOR	GLY	PRO	TIA	TIO	URE	VAL
1	*Candida krusei*	4/25	16	−	−	−	−	−	−	−	−	−	−
1	*Kluyveromyces marxianus*	1/1	100										
2	*Cryptococcus uniguttulatus*	1/1	100	−	−	−	−	−	−	−	−	+	−
3	*Candida albicans*	298/298	100	−	−	−	−	−	+	−	−	−	−
3	*Candida glabrata*	28/28	100										
3	*Candida krusei*	15/25	60										
3	*Pichia membranaefaciens*	1/1	100										
4	*Candida krusei*	3/25	12	−	−	+	−	−	−	−	−	−	−
5	*Cryptococcus neoformans*	2/2	100	−	−	−	−	−	+	−	−	+	−
6	*Candida krusei*	3/25	12	−	−	+	−	−	+	−	−	−	−
6	*Candida inconspicua*	2/2	100										
6	*Pichia anomala*	2/2	100										
6	*Trichosporon capitatum*	1/3	33										
7	*Candida lusitaniae*	1/1	100	+	−	−	−	−	+	−	−	−	−
8	*Candida guilliermondii*	2/2	100	+	−	−	+	−	+	−	−	−	−
9	*Trichosporon capitatum*	2/3	66	+	−	+	−	−	+	−	−	−	−
10	*Candida tropicalis*	70/70	100	+	−	−	+	−	+	−	−	−	+
11	*Geotrichum candidum*	2/2	100	+	−	+	+	−	+	+	−	−	−
12	*Rhodotorula glutinis*	7/7	100	+	−	+	+	−	+	−	−	+	+
12	*Rhodotorula mucilaginosa*	3/3	100										
13	*Candida parapsilosis*	6/47	13	+	−	+	+	+	−	−	−	−	+
14	*Candida parapsilosis*	41/47	87	+	−	+	+	+	+	−	−	−	+
15	*Trichosporon beigelii*	4/4	100	+	+	+	+	+	+	+	+	+	−
16	*Candida lipolytica*	1/1	100	+	+	+	+	+	+	−	+	+	−

ACE, acetamide; ACR, acrylamide; ALA, alaninamide; FOR, formamide; GLY, glycinamide; PRO, propionamide; TIA, thioacetamide; URE, urea; VAL, valeramide.

ide (FOR), glycinamide (GLY), propionamide (PRO), thioacetamide (TIA), thiourea (TIU), urea (URE) and valeramide (VAL). A total of 16 amidase patterns were distinguished, four of them shared by more than one species. *Candida krusei* strains displayed four different patterns, the most numerous of which was exhibited by 60% of strains. *Candida parapsilosis* and *Trichosporon capitatum* strains exhibited two patterns, one preferential and the other secondary. These were the only species to present more than one pattern. The variations in *Candida krusei* were: hydrolysis of propionamide negative in 28% of strains and hydrolysis of alaninamide positive in 24% of strains. The other variations were: negative propionamide in 12.8% of *C. parapsilosis* strains and negative acetamide in 33.3% of *T. capitatum*. From a taxonomic standpoint two amides, thioacetamide (TIA) and thiourea (TIU), may be dropped from the list without affecting the results. Table 3, in which all the species assayed (except *Rhodotorula* strains) can be identified, was drawn up from activity against the remaining amides and the results of three classic tests: formation of a surface film on liquid medium (PEL), tolerance of cycloheximide (ACT) and ascospore formation (ASC).

Discussion

The 500 yeasts strains identified in this study represent most of the genera that are of recognized clinical interest, with the exception of the genus *Saccharomyces*, of which no isolate was identified. Some of the species detected, such as *Candida inconspicua*, *C. lipolytica*, *Cryptococcus uniguttulatus*, *Kluyveromyces marxianus*, *Pichia anomala* and *Pichia membranaefaciens*, are uncommon, either because they rarely occur in our clinics or because they sometimes escape detection owing to lack of adequate methods. It is not easy to characterize them adequately without additional tests not included in commercial systems in regular use. This indicates the difficulty sometimes involved in identifying yeasts, particularly when species are in a teleomorphic state. Examination of amidase activity showed marked differences between most of the strains and enabled them to be grouped by amidase spectrum. On the 16 profiles identified, only four were shared by more than one species, although in one case this included *Rhodotorula glutinis* and *Rhodotorula mucilaginosa*, species which share many characteristics. *C. krusei* strains proved to be the most difficult to identify, presenting four

Table 3. Short amidasic patterns of 500 yeast strains and results of three complementary differential tests

Species	Amidasic patterns								Complementary tests		
	ACE	ACR	ALA	FOR	GLY	PRO	URE	VAL	PEL	ACT	ASC
C. krusei	−	−	−	−	−	−	−	−	+	−	−
K. marxianus									−	+	+
Cr. uniguttulatus	−	−	−	−	−	−	+	−			
C. krusei	−	−	+	−	−	−	−	−			
C. parapsilosis	+	−	+	+	+	−	−	+			
C. albicans	−	−	−	−	−	+	−	−	−	+	−
C. glabrata									−	−	−
C. krusei									+	−	−
P. membranaefaciens									+	−	+
Cr. neoformans	−	−	−	−	−	+	+	−			
C. krusei	−	−	+	−	−	+	−	−	+	−	−
C. inconspicua									−	−	−
P. anomala									−	−	+
T. capitatum									+	+	−
C. lusitaniae	+	−	−	−	−	+	−	−			
C. guilliermondii	+	−	−	+	−	+	−	−			
C. tropicalis	+	−	−	+	−	+	−	+			
T. capitatum	+	−	+	−	−	+	−	−			
G. candidum	+	−	+	+	−	+	−	−			
T. beigelii	+										
C. lipolytica	+	+	+	+	+	+	+	−			
R. glutinis	+	−	+	+	−	+	+	+			
R. mucilaginosa											
C. parapsilosis	+	−	+	+	+	+	−	+			

ACE, acetamide; ACR, acrylamide; ALA, alaninamide; FOR, formamide; GLY, glycinamide; PRO, propionamide; URE, urea; VAL, valeramide; PEL, surface film formation on liquid medium; ACT, tolerance of cycloheximide; ASC, ascospore formation.

separate profiles, three of which were shared by other species. On the sole basis of hydrolysis of eight amides, we succeeded in identifying the species of all isolates, with the assistance on occasions of two classic tests which are in routine use and are easy to apply: formation of a surface film on a liquid medium and tolerance of cycloheximide. Examination of ascospore formation was necessary for recognition of yeasts in teleomorphic state. Only in the case of *Rhodotorula* did other tests prove necessary, such as reduction of nitrates or growth in 50% glucose. Figures 1 and 2 show dendrograms obtained by numerical taxonomy of the 500 yeast strains. The first was compiled from

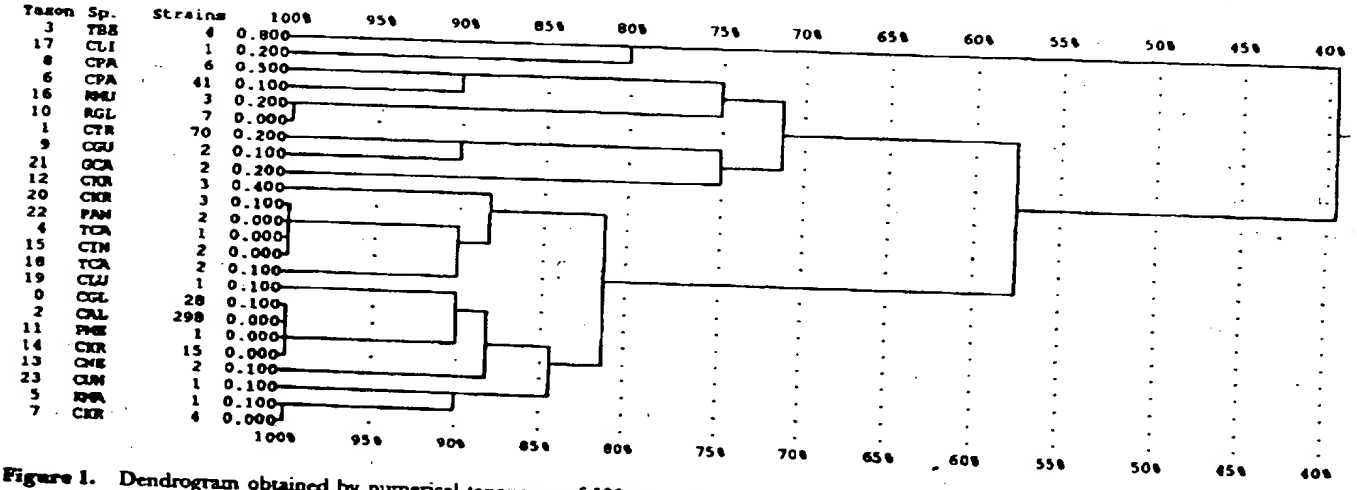

Figure 1. Dendrogram obtained by numerical taxonomy of 500 yeast strains and their amidase activity against 23 amides: simple matching coefficient and Group-average clustering. $r=0.876$. CAL, *Candida albicans*; CGL, *Candida glabrata*; CGU, *Candida guilliermondii*; CIN, *Candida inconspicua*; CKR, *Candida krusei*; CLI, *Candida lipolytica*; CLU, *Candida lusitaniae*; CPA, *Candida parapsilosis*; CTR, *Candida tropicalis*; CUN, *Cryptococcus uniguttulatus*; CNE, *Cryptococcus neoformans*; GCA, *Geotrichum candidum*; KMA, *Kluyveromyces marxianus*; PAN, *Pichia anomala*; PME, *Pichia membranaefaciens*; RGL, *Rhodotorula glutinis*; RMU, *Rhodotorula mucilaginosa*; TBE, *Trichosporon beigelii*; TCA, *Trichosporon capitatum*.

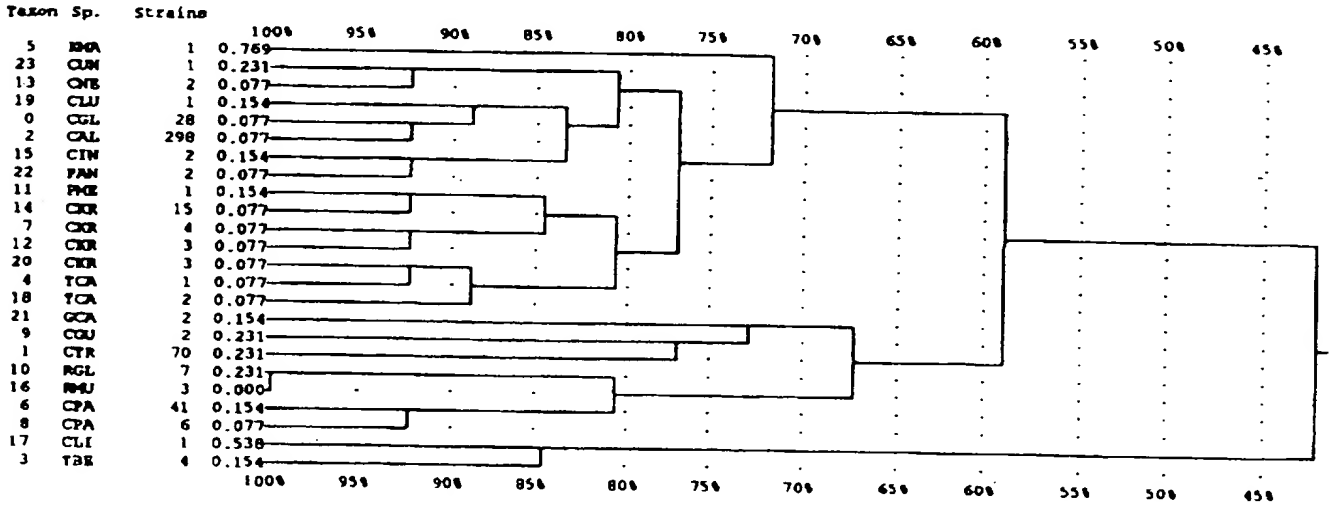

Figure 2. Dendrogram obtained by numerical taxonomy of 500 yeast strains and their amidase activity against 23 amides and results of three complementary tests: simple matching coefficient and group-average clustering. $r = 0.838$. For abbreviations, see Fig. 1.

the profile of activity against the 23 amides and the second with the addition of the three supplementary conventional tests (PEL, ACT and ASC). The mathematical basis for both is the simple matching coefficient and group-average clustering [19, 20]. Dendrogram 1 (Fig. 1) misidentifies taxa 16 (RMU) and 10 (RGL), taxa 20 (CKR), 22 (PQN), 4 (TCA) and 15 (CIN) and also taxa 0 (CGL), 2 (CAL), 11 (PME and 14 (CKR) and even taxa 5 (KMA) and 7 (CKR). After the addition of the three complementary tests, dendrogram 2 (Fig. 2) shows the identification of these taxa as species, except taxa 10 (RGL) and 16 (RMU), and the new order of taxa. The phenetic relations between species in dendrogram 2 (Fig. 2) are very evident.

The capacity of yeasts and yeast-like fungi to hydrolyse amides has not been applied hitherto as a taxonomical criterion, except in the case of the *Cryptococcus* genus. In our experience the amidase spectrum applied in this work has proved a valid tool for identification of yeasts, whose incorporation into routine practice can help to simplify currently available identification procedures, to improve identification of species and biovars and to economize on the use of resources in clinical microbiological practice.

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